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REVIEW

Parsing the players: 2-arachidonoylglycerol synthesis and degradation in the CNS

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The endogenous cannabinoid signalling system, composed of endogenous cannabinoids, cannabinoid receptors and the enzymes that synthesize and degrade the endogenous cannabinoids, is much more complex than initially conceptualized. 2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid and plays a major role in CNS development and synaptic plasticity. Over the past decade, many key players in 2-AG synthesis and degradation have been identified and characterized. Most 2-AG is synthesized from membrane phospholipids via sequential activation of a phospholipase Cβ and a diacylglycerol lipase, although other pathways may contribute in specialized settings. 2-AG breakdown is more complicated with at least eight different enzymes participating. These enzymes can either degrade 2-AG into its components, arachidonic acid and glycerol, or transform 2-AG into highly bioactive signal molecules. The implications of the precise temporal and spatial control of the expression and function of these pleiotropic metabolizing enzymes have only recently come to be appreciated. In this review, we will focus on the primary organization of the synthetic and degradative pathways of 2-AG and then discuss more recent findings and their implications, with an eye towards the biological and therapeutic implications of manipulating 2-AG synthesis and metabolism.

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Abbreviations
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABHD6, serine hydrolase α-β-hydrolase domain 6; ABHD12, serine hydrolase α-β-hydrolase domain 12; AEA, anandamide; CaMKII, calcium calmodulin kinase II; DAGL, diacylglycerol lipase; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; FAAH, fatty acid amide hydrolase; LPA, lysophosphatidic acid; LTD, long-term depression; lyso-PLC, lyso phospholipase C; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; mGluR, metabotropic glutamate receptor; MSE, metabotropic suppression of excitation; PIP_2, phosphatidylinositol 4,5-bisphosphate; THC, Δ^9-tetrahydrocannabinol

Introduction

Humans have used *Cannabis sativa* for at least 8000 years for recreational and therapeutic purposes (Zuardi *et al.*, 2006). Its chief psychoactive component is Δ^9-tetrahydrocannabinol (THC), which was chemically characterized in 1965 (Mechoulam and Gaoni, 1965). THC engages several receptors, including the cannabinoid CB_1 and CB_2 receptors (receptor nomenclature follows Alexander *et al.*, 2013) These receptors are a part of the endogenous cannabinoid (endocannabinoid) signalling system, which regulates analgesia, memory, synaptic plasticity, learning, appetite, peripheral metabolism, immune function and many other physiological processes (Hohmann *et al.*, 1995; Di Marzo *et al.*, 1998; Cravatt *et al.*, 2001; Brenowitz and Regehr, 2005; Kishimoto and Kano, 2006). Thus, understanding endocannabinoid signalling is pivotal to understanding the complex networks regulating these diverse processes.

The gene for the first cannabinoid receptor, CB_1, was cloned in 1990 (Matsuda *et al.*, 1990). This receptor is found throughout the nervous system (Herkenham *et al.*, 1990) and is also present in certain cells of the immune system, adipose

tissue, liver, muscle, reproductive cells, kidney and lungs (Pagotto *et al.*, 2006). CB_1 receptors belong to the GPCR family (Mukhopadhyay and Howlett, 2001), are involved in regulating many neuronal networks (Kano *et al.*, 2009) and are the most highly expressed GPCRs in the brain (Devane *et al.*, 1988; Herkenham *et al.*, 1990).

The gene for the second cannabinoid receptor, CB_2, was cloned in 1993. These receptors have 44% amino acid homology to the CB_1 receptor (Munro *et al.*, 1993) and are most abundant in immune cells and their descendants (Galiegue *et al.*, 1995). However, the extent of CB_2 receptor expression is unclear and the precise distribution of CB_2 receptors, especially in the nervous system, is still debated (Atwood *et al.*, 2012).

The endocannabinoids are the signalling components of the endocannabinoid system. Although peptides that interact with CB_1 receptors (the pepcans; Bauer *et al.*, 2012), including the peptide hemopressin (Heimann *et al.*, 2007), have been described, the focus of this review will be on the synthesis and degradation of lipid endocannabinoids, particularly 2-arachidonoylglycerol (2-AG). The endocannabinoids bind to CB receptors, but vary in their affinity, efficacy and metabolism. The two most studied endocannabinoids are N-arachidonoyl ethanolamine, also known as anandamide (AEA), and 2-AG. Lipid endocannabinoids are membrane-preferring; they can diffuse across membranes but do not participate in vesicle-mediated release. As a consequence, endocannabinoids are thought to be synthesized enzymically 'on demand' from lipid precursors (Di Marzo *et al.*, 1998). However, some evidence suggests that in certain cases, 2-AG might be preformed and sequestered until needed (Alger and Kim, 2011). This possibility is based on the observation that inhibitors of the main synthetic enzyme for 2-AG are sometimes unable to block 2-AG-dependent responses (Chevaleyre and Castillo, 2003). However, a recent paper challenges the notion of preformed 2-AG pools, making the argument that experimental conditions may explain the discrepancies (Hashimotodani *et al.*, 2013).

2-AG has been implicated in a wide variety of physiological processes, including several forms of neuroplasticity (Kano *et al.*, 2009). In addition to its signalling roles, 2-AG is also an important intermediate in lipid metabolism (Ahn *et al.*, 2008). Thus, measurement of 2-AG from tissue samples represents both 'signalling' and 'metabolic-intermediate' levels of 2-AG and it is likely that only a small fraction of the 2-AG measured in tissue samples is functioning as an endocannabinoid (Caille *et al.*, 2007). The synthesis of 2-AG appears to occur through relatively few pathways, but its degradation is more complex. The best studied synthetic pathways for 2-AG are its synthesis from diacylglycerols (DAG) with arachidonic acid at the 2-position (Stella *et al.*, 1997) by one of two diacylglycerol lipases (DAGL) – DAGLα and DAGLβ (Bisogno *et al.*, 2003). In addition, 2-AG can also be synthesized by dephosphorylation of arachidonoyl-LPA (Nakane *et al.*, 2002) or by the sequential action of PLA1 and a lyso phospholipase C (lyso-PLC) (Higgs and Glomset, 1994).

The metabolism of 2-AG is more complicated in that several enzymes, operating in different compartments and contexts, are involved. Enzymes metabolising 2-AG are located both post-synaptically and pre-synaptically (Blankman *et al.*, 2007; Kano *et al.*, 2009; Straiker *et al.*, 2011). This provides several parallel mechanisms for spatial control of endocannabinoid signalling. Additional spatial specificity may be imparted by the subcellular localization of the enzymes as GPCR signalling is often restricted to certain organelles (Caille *et al.*, 2007). Monoacylglycerol lipase (MAGL) is considered to be the chief 2-AG degrading enzyme, but at least three other serine hydrolases also contribute: fatty acid amide hydrolase (FAAH), serine hydrolase α-β-hydrolase domain 6 (ABHD6) and serine hydrolase α-β-hydrolase domain (ABHD12) (Blankman *et al.*, 2007). All of these pathways lead to two major 2-AG breakdown products: arachidonic acid (AA) and glycerol (Freund *et al.*, 2003). Additional routes of 2-AG metabolism produce new signalling molecules. For example, COX-2 oxidizes 2-AG under certain circumstances (Straiker *et al.*, 2011), producing prostaglandin glycerol esters (Sang *et al.*, 2007; Hu *et al.*, 2008; Richie-Jannetta *et al.*, 2010). Phosphorylation of 2-AG by acyl glycerol kinase(s) creates lysophosphatidic acid (LPA) (Bektas *et al.*, 2005), which activates different signalling pathways (Moolenaar *et al.*, 1997). Finally, lipoxygenases can oxidize 2-AG, producing hydroperoxy derivatives of 2-AG (Kozak and Marnett, 2002). The bioactive role of these latter 2-AG metabolites is often opposite to that of 2-AG (e.g. excitatory rather than inhibitory). Therefore, inhibiting the metabolic enzymes can have profound cellular consequences. The complexity of 2-AG regulatory mechanisms (Figure 1) is considerable and deserves a closer examination. This review summarizes recent major discoveries in the areas of 2-AG synthesis and metabolism in the CNS.

Three major pathways for 2-AG synthesis

Three major pathways have been proposed for 2-AG synthesis (Figure 2). The first is the production of 2-AG via a two-step process, starting with phosphatidylinositol 4,5-bisphosphate (PIP_2) proceeding via a DAG intermediate to 2-AG (Farooqui *et al.*, 1989). The first step is catalysed by a phospholipase C-β (PLCβ) (Farooqui *et al.*, 1989), whereas the second step is catalysed by one of two DAGLs (Bisogno *et al.*, 2003; Tanimura *et al.*, 2010). This pathway appears to dominate in the CNS (Kano *et al.*, 2009). The second pathway involves the conversion of phosphatidyl lipid (e.g. PI) to 2-arachidonoyl-lyso PI, by the action of a PLA1, and then to 2-AG by the action of lyso-PLC (Higgs and Glomset, 1994) (Figure 2). The third pathway involves LPA hydrolysis by an LPA phosphatase (Nakane *et al.*, 2002). The involvement of these latter two pathways in the production of 2-AG in the CNS has not been evaluated in detail but may account for some reports of endocannabinoid-mediated synaptic plasticity that is insensitive to DAGL inhibitors (Zhang *et al.*, 2011).

Two DAGL isoforms produce 2-AG

Two DAGL isoforms have been identified – DAGLα and DAGLβ (Bisogno *et al.*, 2003). DAGLs are highly conserved between species, with human and mouse DAGLα sharing 97% homology and the DAGLβs sharing 79% homology. The

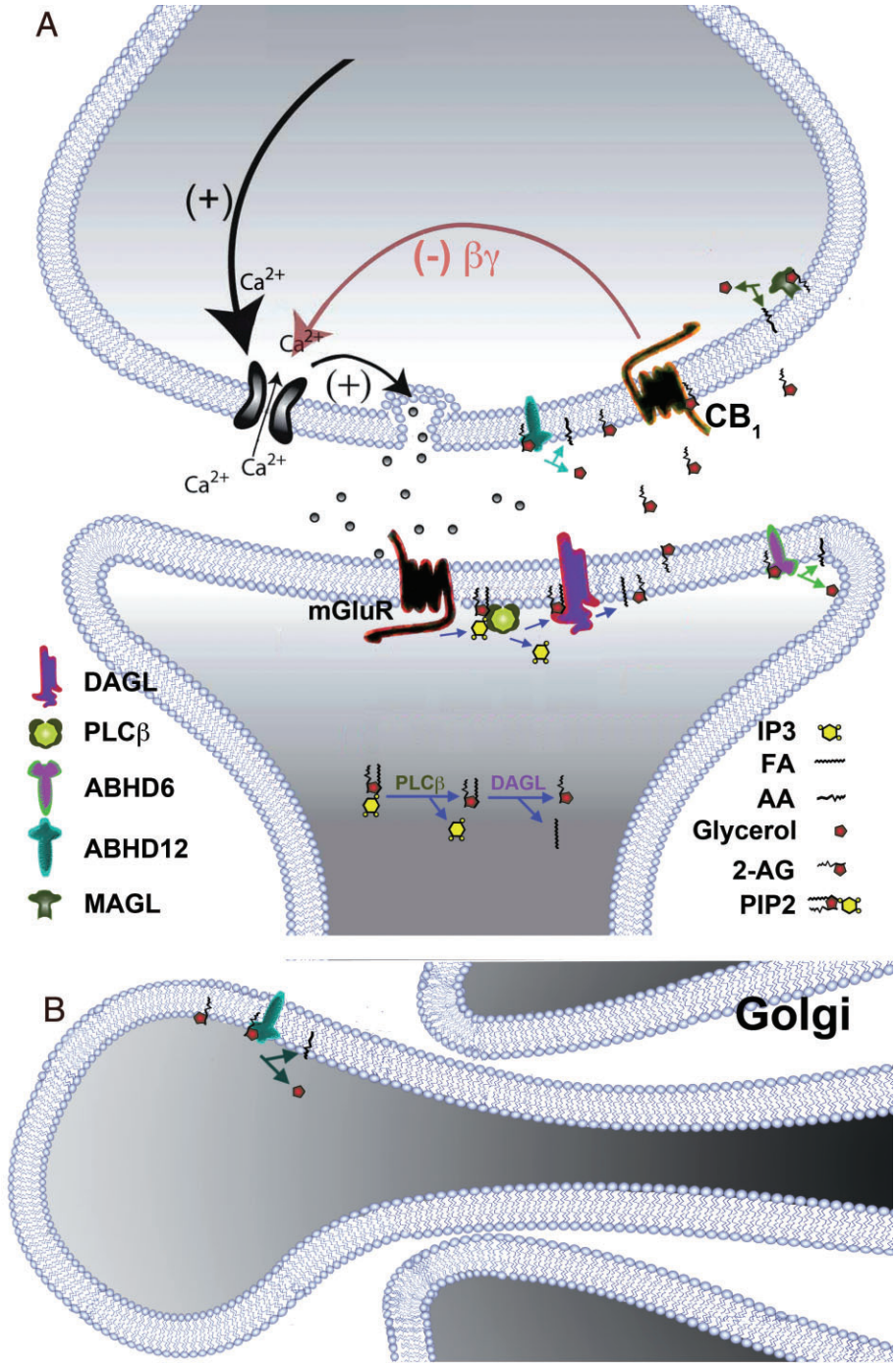

Figure 1

2-AG trafficking and its action at the synapse. (A) 2-AG synthesis and breakdown. Schematic representation of an excitatory terminal and post-synaptic spine. Conventional action potential-induced neurotransmitter release (e.g. glutamate) occurs via activation of calcium channels adjacent to transmitter-filled vesicles, which fuse with the membrane to release their contents. 2-AG can be produced either following synaptic depolarization (e.g. DSE) or by activation of $G_{q/11}$-coupled GPCRs, such as group I mGlu receptors, which then activate PLCβ (PLCβ, cleaving phosphatidyl bisphosphate (PIP_2) into DAG and inositol trisphosphate (IP_3). DAG is hydrolysed by DAG lipase, yielding 2-AG. Rather than being released from vesicles, lipophilic endocannabinoids cross the membrane, perhaps utilizing facilitated transport. The mechanism of subsequent passage across the synapse is unknown but may involve carrier proteins. Activation of pre-synaptic CB_1 receptors inhibits transmitter release by inhibiting Ca^{2+} channels. On the post-synaptic side, 2-AG can be broken down into glycerol and AA by the enzyme ABHD6, embedded in the membrane. On the pre-synaptic side, 2-AG can be broken down by MAGL, loosely associated with the plasma membrane, or, in principle, by ABHD12, a transmembrane protein, into glycerol and AA. (B) ABHD12 localization on Golgi. Emerging evidence suggests that ABHD12 is embedded in the Golgi membrane, with its active site facing the lumen.

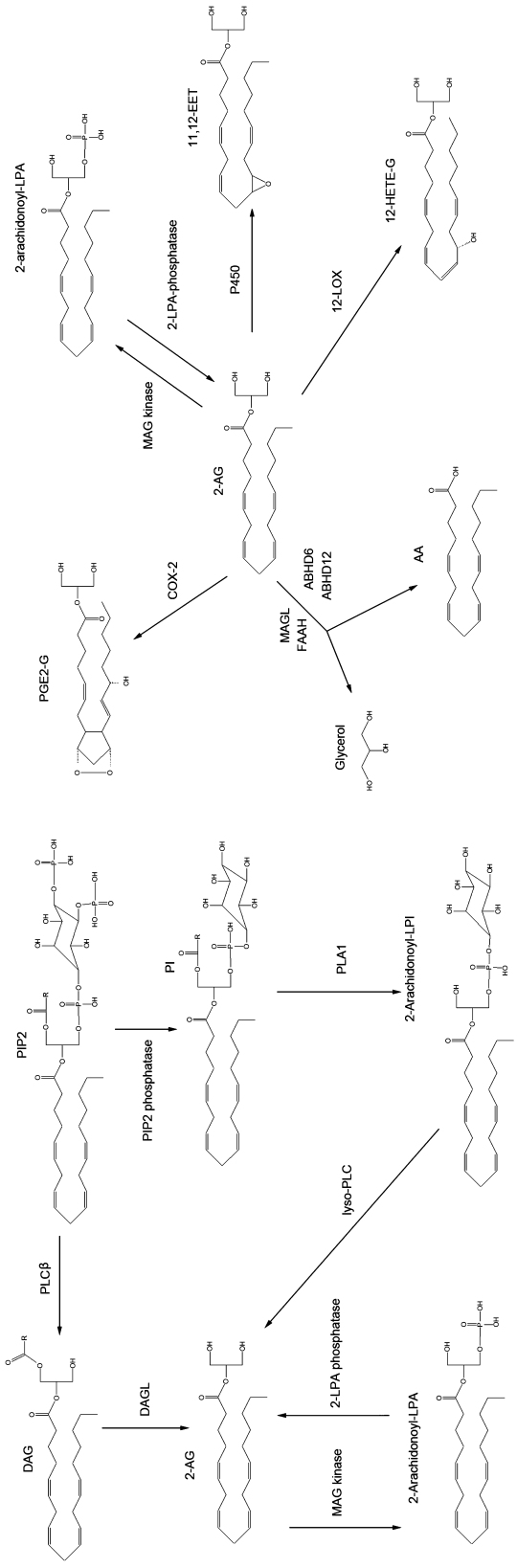

Figure 2

Primary routes of 2-AG synthesis and degradation. 2-AG synthesis can potentially occur from three precursors: (1) DAG (via DAGL), in turn, DAG can be synthesized from PIP_2 via PL C-β (PLCβ); (2) 2-Arachidonoyl-LPA via 2-LPA phosphatase (MAG kinase reverses the actions of 2-LPA phosphatase); and (3) 2-Arachidonoyl-LPI via a lyso-PLC, 2-Arachidonoyl-LPI can, in turn, be synthesized from PI via the actions of PLA1 (PIP_2 phosphatases produce PI from PIP_2). 2-AG metabolism can potentially occur via five different routes: (1) MAGL, FAAH, ABHD6 and ABHD12 hydrolyse 2-AG into AA and glycerol; (2) COX-2 converts 2-AG into PGE_2-G; (3) MAG kinase converts 2-AG into 2-arachidonoyl-LPA (2-LPA phosphatase reverses the actions of MAG kinase); (4) cytochrome p450 converts 2-AG into 11,12-EET and (5) 12-lipoxygenase (12-LOX) can oxygenate 2-AG into 12-HETE-G.

two enzymes differ from each other by the presence of a long C-terminal tail (~300 amino acids) in DAGLα relative to DAGLβ (Oudin *et al.*, 2011). The C-terminal tail in DAGLα facilitates its regulation by calcium calmodulin kinase II (CaMKII), which phosphorylates serine residues in this region, decreasing DAGLα activity (Shonesy *et al.*, 2013). In mice expressing an activation-impaired form of CaMKII (T268A), both DAGL activity and 2-AG production are increased (Shonesy *et al.*, 2013). Homer, an important post-synaptic scaffolding protein (Szumlinski *et al.*, 2006), appears to be involved in targeting of DAGLα towards group I metabotropic glutamate receptors (mGluRs) in dendritic spines (Jung *et al.*, 2007; Oudin *et al.*, 2011). Moreover, in murine DAGLα, the amino acid residues 973–980 comprise a consensus motif, PPxxF, for binding the coiled-coil domain of homer proteins (Jung *et al.*, 2007). Interestingly, homer 1a, which lacks the ability to partner with other homers and disrupts synaptic scaffolding, decreases group I mGluR-mediated synaptic plasticity, while enhancing depolarization-induced suppression of excitation (DSE) (Roloff *et al.*, 2010), emphasizing the importance of DAGL's spatial localization in fine-tuning synaptic plasticity.

Localization of DAGLα and DAGLβ appears to be complementary and tissue-specific

DAGLα mRNA is expressed throughout the brain, but most notably in the hippocampus, striatum, ventral tegmental area and cerebellum; DAGLβ's expression pattern is not as well characterized (Oudin *et al.*, 2011). The highest levels of DAGLα mRNA are found in hippocampal pyramidal cells, dentate granule cells and cerebellar Purkinje cells. In contrast, the highest levels of DAGLβ mRNA are present in the cerebellar granular layer; with low levels in the hippocampal pyramidal cell layer and thalamus (Yoshida *et al.*, 2006). In adult brain, DAGLα is generally found post-synaptically where it is enriched in the plasma membrane of dendritic spines, particularly in the spine neck (Katona *et al.*, 2006; Yoshida *et al.*, 2006; Jung *et al.*, 2007; Ludanyi *et al.*, 2011). Much less is known about the subcellular distribution of DAGLβ, although recent evidence suggests that it is more broadly expressed in dendrites of cultured hippocampal neurons than is DAGLα (Jain *et al.*, 2013). At excitatory synapses, CB_1 receptors and DAGLα are found in close proximity – CB_1 receptors are pre-synaptic and DAGLα is post-synaptic (Katona *et al.*, 2006; Yoshida *et al.*, 2006). This consistent spatial arrangement of 2-AG synthesis and effector sites at excitatory synapses appears to facilitate precise control of 2-AG release towards CB_1 receptors. Importantly, whereas CB_1 receptors and DAGLα are closely apposed at excitatory synapses, CB_1 receptors at inhibitory synapses can be quite distant from detectable DAGLα (Katona *et al.*, 2006; Yoshida *et al.*, 2006).

The distribution of DAGLα and DAGLβ changes markedly during development. In developing mouse forebrain projection neurons, DAGLs are often co-expressed with CB_1 receptors in elongating axons (Bisogno *et al.*, 2003; Mulder *et al.*, 2008; Keimpema *et al.*, 2010; Wu *et al.*, 2010). However, postnatally CB_1 receptors concentrate in axon terminals, whereas the DAGLs accumulate in dendrites (Keimpema *et al.*, 2011). In summary, both DAGLα and DAGLβ are widely distributed. Prenatally, both tend to be expressed in axons, whereas postnatally they are found in dendrites and dendritic spines.

DAGLα and DAGLβ function

Studies using pharmacological inhibition of DAGLs implicate these enzymes in 2-AG-mediated synaptic plasticity (Kano *et al.*, 2002; 2009). However, these inhibitors do not distinguish between DAGLα and DAGLβ, making it impossible to determine which of the two DAGLs is involved in 2-AG production. The development of three independent lines each of DAGLα and DAGLβ knockout (KO) mice has helped in this regard (Gao *et al.*, 2010; Tanimura *et al.*, 2010; Yoshida *et al.*, 2011). All reported essentially the same finding: the absence of DAGLα eliminates every form of synaptic plasticity examined. These results appeared to fully resolve the question of which DAGL mediates synaptic plasticity in favour of DAGLα, although it leaves unresolved the role of the relatively abundant DAGLβ in neurons.

If a DAGL plays a major role in 2-AG synthesis, 2-AG levels would be predicted to decline in the corresponding DAGL KO mice. Indeed, Tanimura *et al.* (2010) found significant decreases in 2-AG levels in both DAGLα–/– and DAGLβ–/– mice. However, there were major differences among tissues. For example, in the CNS of DAGLα–/– mice, there is an 80% decrease in 2-AG levels and a 50% decrease in 2-AG levels in the CNS of DAGLβ–/– mice. However, in the liver of DAGLβ–/– mice, there is an ~90% reduction in 2-AG levels and in the DAGLα–/– mice 2-AG is only reduced by 50% (Gao *et al.*, 2010). This underscores the notion that either enzyme may be important for 2-AG production in a tissue-dependent fashion. For example, an ethanol-rich diet up-regulates hepatic DAGLβ expression, leading to steatosis (Jeong *et al.*, 2008) and DAGLβ mediates certain pro-inflammatory responses in peritoneal macrophages (Hsu *et al.*, 2012). Cerebral morphology was unaffected in either DAGLα or DAGLβ KO mice (Tanimura *et al.*, 2010). Functionally, however, adult neurogenesis in the dentate gyrus and sub-ventricular zone was diminished in both DAGLα–/– and DAGLα+/– mice, but lack of DAGLβ had no measurable effect (Gao *et al.*, 2010). Loss of one DAGL did not affect mRNA for the other DAGL nor did it alter the levels of MAGL, FAAH, CB_1 or CB_2 receptor mRNA (Gao *et al.*, 2010).

Whereas these experiments strongly argue for an exclusive role for DAGLα in endocannabinoid-mediated synaptic plasticity, KO studies using constitutive deletions have limitations insofar as developmental adaptations to the absence of a protein may have long-term or secondary effects that are difficult to predict. For example, in the brain, spinal cord and liver of DAGLα–/– mice, AEA levels are decreased despite this enzyme not having a direct role in AEA synthesis (Gao *et al.*, 2010).

A recent study using RNAi knockdown in cultured hippocampal neurons suggests that DAGLα and DAGLβ can cooperate to mediate two forms of endocannabinoid plasticity: DSE and metabotropic suppression of excitation (MSE) (Jain *et al.*, 2013). In these experiments, DSE was diminished by knockdown of either DAGL and reduced

almost completely when both enzymes were knocked down, consistent with a cooperative effect. Similarly, knockdown of either DAGL decreased MSE mediated by mGluR5. This indicates that at the very least, DAGLβ is capable of eliciting endocannabinoid-mediated synaptic plasticity under some conditions and that the two enzymes can share this function in the same neuron. Hopefully, it will also motivate studies using inducible DAGLα and DAGLβ KO mice to assess more thoroughly the role of these two enzymes in 2-AG production.

In summary, 2-AG production appears to occur chiefly via the two DAGLs, but which isoform is actually responsible still remains to be established. 2-AG function, the location of its metabolic enzymes and CB_1 receptors all undergo a pronounced developmental change from a predominantly pre-synaptic path-finding role in the developing CNS to a primarily post-synaptic neuromodulatory role in the mature CNS. DAGLα activity is highly regulated, including stimulation by increases in intracellular calcium (Bisogno *et al.*, 2003) and increase in substrate (DAG) by activation of PLCβ, and inhibition by CaMKII phosphorylation and disrupted targeting by homer 1a (Jung *et al.*, 2007; Kano *et al.*, 2009; Won *et al.*, 2009; Roloff *et al.*, 2010). Attempts to harness the cannabinoid system for therapeutic purposes have focused on synthetic receptor agonists and antagonists and, to a lesser extent, inhibitors of enzymic breakdown. However, the enzymes that produce and regulate the production of 2-AG may offer alternative targets for particular indications.

When the job is (partially) done: 2-AG metabolism

Much evidence supports the notion that in the mature CNS, 2-AG frequently acts as a retrograde synaptic signal, produced pre-synaptically and acting post-synaptically. As 2-AG traverses the synaptic cleft and reaches the pre-synaptic terminal, it enters into the membrane, where it can bind to the CB_1 receptor. In the pre-synapse, 2-AG can diffuse beyond the CB_1 receptor, possibly engaging other targets. Therefore, its prompt metabolism is important to avoid unintended actions. In addition, 2-AG also serves as an important intermediate in lipid metabolism (Ho and Randall, 2007). Our understanding of 2-AG metabolism has increased in recent years in large part due to the development of specific inhibitors and several lines of KO mice. This section will summarize many of those findings and identify key questions that remain regarding the details of 2-AG metabolism.

2-AG can be broken down or modified by a diverse assortment of enzymes that either hydrolyse it into its component parts (AA and glycerol) or chemically transform it by acylating or phosphorylating the glycerol or oxidizing its AA moiety (Figure 2). Enzymes involved in hydrolysis include MAGL, ABHD6, ABHD12 and FAAH (Blankman *et al.*, 2007). Enzymes involved in chemical transformation include COX-2 (Kozak *et al.*, 2000), cytochrome P450 (Chen *et al.*, 2008), lipoxygenases (Maccarrone *et al.*, 2000), monoacylglycerol (MAG) kinases (Kanoh *et al.*, 1986) and MAG acyl transferases (Coleman and Haynes, 1986).

When considering studies investigating the enzymes involved in 2-AG degradation, it is important to distinguish between results from *in vitro* experiments, which address the question of whether a particular enzyme *can* metabolize 2-AG, and those from *in vivo* experiments that address the question of whether the enzyme has *physiological relevance* in a particular context. It is well accepted that MAGL is the dominant enzyme in degrading 2-AG in its endocannabinoid retrograde messenger role, but at least four other enzymes – ABHD6, ABHD12, FAAH and COX-2 – have important, but more specialized roles in endocannabinoid retrograde signalling. Studies examining 2-AG metabolism raise intriguing questions that we will address below: Which of these enzymes are active members of an endogenous 2-AG-based cannabinoid signalling system? Where are they found and when do they contribute? Do they act cooperatively or in a division of roles? For example, does one enzyme engage in bulk clearance of 2-AG at the pre-synaptic terminal while another breaks down the neurotransmitter on the post-synaptic side? Does their activity level or function depend on the cell type that they are expressed in?

MAGL is responsible for acute breakdown of 2-AG, and more . . .

MAGL is primarily pre-synaptically localized (Gulyas *et al.*, 2004). It contains 302 amino acids, with its catalytic triad, Ser^{122}, Asp^{239} and His^{269} located in turns between α-helices and β-sheets (Karlsson *et al.*, 1997). In addition, MAGL contains an HG-dipeptide (His^{49} and Gly^{50}) motif common to all lipases (Karlsson *et al.*, 2001). Moreover, three cysteine residues, Cys^{242} (King *et al.*, 2009), Cys^{201} and Cys^{208} (Jaeger *et al.*, 1999), regulate MAGL function, as their mutation decreases hydrolytic activity. In addition, molecular and Western blotting evidence supports the existence of several MAGL splice variants (Karlsson *et al.*, 2001). MAGL is localized intracellularly and is found in both soluble and membrane fractions (Blankman *et al.*, 2007). It is pre-synaptic and a loosely membrane-associated location is ideal to break down 2-AG in the proximity of CB_1 receptors. Our understanding of the prominent role of MAGL in degrading 2-AG in the CNS comes from the observation that inhibiting or knocking out MAGL leads to large increases in brain 2-AG levels. For example, in MAGL–/– mice, 2-AG levels increase 58-fold (Taschler *et al.*, 2011). The quantitatively dominant role of MAGL among other serine hydrolases in hydrolysing 2-AG comes from an experiment where 32 serine hydrolases expressed in the brain were tested for their ability to hydrolyse 2-AG. The great majority of 2-AG was hydrolysed by MAGL (Blankman *et al.*, 2007). Evidence for a role of MAGL in terminating the action of retrogradely *released* 2-AG comes from experiments where endocannabinoid (2-AG)-mediated synaptic plasticity is prolonged in slices or cultured neurons prepared from MAGL KO animals (Kano *et al.*, 2009; Straiker and Mackie, 2009).

Complete pharmacological or genetic inactivation of MAGL increases endocannabinoid tone and causes CB_1 receptor desensitization, tolerance to CB_1 receptor agonists and down-regulation of CB_1 receptors (Chanda *et al.*, 2010; Schlosburg *et al.*, 2010). These findings emphasize the importance of MAGL as an enzyme playing a major role in

hydrolysing synaptic 2-AG. An important practical question is how much MAGL inhibition is necessary for CB_1 receptor desensitization and is there a therapeutic window where pharmacologically useful MAGL inhibition occurs, without desensitization of CB_1 receptor signalling?

2-AG hydrolytic activity in brain membranes from MAGL mice lacking just one allele (MAGL+/–) was about half of that of wild-type mice (Schlosburg *et al.*, 2010). 2-AG levels in these animals were also significantly elevated (about twice over wild-type levels) (Schlosburg *et al.*, 2010). The twofold increase in 2-AG levels did not significantly desensitize CB_1 receptors (Schlosburg *et al.*, 2010). This suggests that MAGL activity can be significantly reduced and 2-AG levels mildly elevated, before CB_1 receptor desensitization occurs. A number of investigators have studied the effects of chronic MAGL inhibition by JZL184 in mice (Busquets-Garcia *et al.*, 2011; Kinsey *et al.*, 2011; Sumislawski *et al.*, 2011; Ghosh *et al.*, 2013). These studies suggest that chronic (typically ~1 week) parenteral dosing of up to and including 8 $mg \cdot kg^{-1} \cdot day^{-1}$ of JZL184 does not lead to behavioural tolerance or CB_1 receptor desensitization (Busquets-Garcia *et al.*, 2011; Kinsey *et al.*, 2011; Ghosh *et al.*, 2013). However, a chronic JZL184 dose of 16 $mg \cdot kg^{-1} \cdot day^{-1}$ reliably produces tolerance (Sumislawski *et al.*, 2011; Ghosh *et al.*, 2013). Whereas there may be variation between tissues and behaviours, there appears to be a therapeutic window (between 8 and 16 $mg \cdot kg^{-1}$ of JZL184) of MAGL inhibition, where behavioural efficacy is intact and CB_1 receptor signalling is maintained.

Interestingly, in both MAGL KO mice and animals treated with a MAGL inhibitor, in addition to the expected increase in 2-AG brain levels, there was a profound decrease in levels of free AA (Long *et al.*, 2009). It is likely that inhibition of 2-AG degradation via MAGL attenuates AA-based lipid production. Consistent with this idea, several eicosanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane B_2, were decreased (Nomura *et al.*, 2011). Interestingly, MAGL inhibitors prevented the rise in brain eicosanoids and inflammatory cytokines seen following LPS injection, without affecting basal cytokine levels. Moreover, the reduction in activated cytokines was not reversed by CB_1 receptor antagonists, but was mimicked by COX-1 blockade (Nomura *et al.*, 2011), suggesting they were due to COX-1 metabolites and not CB_1 receptor activation. These findings suggest that MAGL inhibition may be an effective therapeutic option for neuroinflammatory conditions. Thus, MAGL is an important enzyme both in regulating synaptic 2-AG-based signalling as well as in controlling brain eicosanoid production.

ABHD6: a post-synaptic guard against 2-AG overproduction?

ABHD6 first drew attention as a 2-AG metabolizing enzyme when it was found to account for a portion (~5%) of estimated 2-AG hydrolase activity in mouse brain (Blankman *et al.*, 2007). ABHD6 is an integral membrane protein with its active site facing the interior of the cell (Blankman *et al.*, 2007). Homology modelling suggests that the first nine residues are extracellular, followed by 30 transmembrane residues and 290 intracellular residues (Bowman and Makriyannis, 2013). In humans, ABHD6 mRNA is found throughout the body, including the brain, the liver, the kidney and the ovary (Li *et al.*, 2009). Prefrontal cortex has high levels of ABHD6, where it is often expressed post-synaptically on the cell membrane of dendritic spines, apposed to immunoreactive CB_1 receptors (Marrs *et al.*, 2010). ABHD6 was also expressed post-synaptically in cultured hippocampal neurons and glia (Straiker *et al.*, 2009; Marrs *et al.*, 2010). Quantitative immunogold labelling found that more than 90% of ABHD6 immunoreactivity in mouse prefrontal cortex was post-synaptic, often in dendritic spines (Marrs *et al.*, 2010). In mouse retina, ABHD6 immunostaining was present in the inner plexiform layer, the inner nuclear layer and the ganglion cell layer. This staining also appeared to be concentrated in calbindin-positive and GAD67-positive amacrine cells, and also co-localized with a dendritic marker (Hu *et al.*, 2010). Thus, ABHD6 has a relatively widespread distribution, primarily as a post-synaptic protein, contrasting with the pre-synaptic localization of MAGL.

A functional role for ABDH6 in 2-AG hydrolysis is apparent in BV-2 cells (a microglial cell line), which lack MAGL, yet efficiently hydrolyse 2-AG (Muccioli *et al.*, 2007). In these cells, ABHD6 was found to be a significant 2-AG metabolizing enzyme, responsible for about half of 2-AG hydrolysis (Marrs *et al.*, 2010). Additionally, roles for ABHD6 in 2-AG hydrolysis have been reported in lysates of cultured cortical cells and intact cultured cortical neurons (Marrs *et al.*, 2011).

Inhibition of ABHD6 or MAGL in prefrontal cortical slices enabled a subthreshold long-term depression (LTD) stimulus to produce LTD via a CB_1 receptor-dependent mechanism. However, the effects of ABHD6 and MAGL inhibition were not additive – blocking both ABHD6 and MAGL did not further increase LTD. Because its active site is predicted to be intracellular, it has been proposed that ABHD6 limits intracellular 2-AG accumulation (Marrs *et al.*, 2010). Interestingly, even though cultured autaptic hippocampal neurons expressed immunoreactive ABHD6, neither ABHD6 inhibition nor its overexpression affected DSE in these neurons (Straiker *et al.*, 2009). Similarly, ABHD6 inhibition did not alter the time course of depolarization-induced suppression of inhibition (DSI) in cortical slices (Marrs *et al.*, 2010). Its dendritic localization and more limited effects on endocannabinoid-mediated synaptic plasticity may indicate that ABHD6 is most relevant during prolonged 2-AG release (e.g. during an LTD stimulus) or when excessive 2-AG levels are reached.

ABHD12: role in an inherited neurological disorder and a specialized means of 2-AG degradation

ABHD12 accounted for about 9% of estimated 2-AG serine hydrolase activity in the mouse brain (Blankman *et al.*, 2007). ABHD12 is predicted to be an integral membrane protein with its active site facing the lumen/extracellular space (Blankman *et al.*, 2007). In cultured hippocampal neurons, ABHD12 immunoreactivity co-localizes with the Golgi apparatus as detected by the lectin, GS-II (Straiker *et al.*,

2013). Moreover, in contrast with other 2-AG hydrolysing enzymes, ABHD12 has been linked to a neurodegenerative disease in humans characterized by polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataracts (PHARC; Fiskerstrand *et al.*, 2010). This disorder is due to loss-of-function mutations of the ABHD12 gene. ABHD12 KO mice have recently been generated (Blankman *et al.*, 2013) and these mice appear normal at a young age, but go on to develop a number of pathologies later in life that resemble PHARC. However, the available evidence suggests that 2-AG hydrolysis by ABHD12 is unrelated to the deficits noted in ABHD12 KO mice. The evidence for this is that 2-AG levels were not significantly altered in these KO mice and 2-AG hydrolysis activity in brain protein homogenates of ABHD12 KO and wild-type mice were comparable. However, pretreatment of the brain protein homogenates with the selective MAGL inhibitor, JZL184 (1 μM), reduced 2-AG hydrolase activity in ABHD12 KO animal homogenates significantly more than in wild-type brain homogenates (Blankman *et al.*, 2013). One interpretation of these data is that ABDH12 is an accessory enzyme that is recruited only when particularly high amounts of 2-AG are present or MAGL is unavailable. In young ABHD12 KO mice, very long chain lysophosphatidylserine (lysoPS) lipids were significantly elevated, prior to the onset of neuroinflammatory and behavioural defects. This correlated with an increase in activated microglia in cerebellar sections of asymptomatic ABHD12 KO mice (Blankman *et al.*, 2013). These data suggest that ABHD12 metabolizes very long chain lysoPS lipids, but has little effect on basal 2-AG levels, at least at the global level. Consistent with these observations, overexpression of ABHD12 in autaptic hippocampal neurons did not shorten the duration of DSE, suggesting that in this system, ABHD12 does not contribute to degrading 2-AG in its role as a retrograde messenger (Straiker *et al.*, 2011). However, knockout of ABHD12 did slightly attenuate EPSC inhibition after the longest depolarization, which corresponded to the largest 2-AG release, suggesting that in the case of 2-AG overproduction, ABHD12 might be involved in its breakdown. Moreover, with increasing time in culture, desensitization of CB_1 receptors in ABHD12 KO neurons occurred, supporting a role of ABHD12 in 2-AG clearance under certain conditions (Straiker *et al.*, 2013).

COX-2 oxygenates 2-AG to produce pro-inflammatory prostaglandin glycerol esters

COX-2 is a prostaglandin-endoperoxide synthase that is essential in the synthesis of prostaglandins from free AA (Xie *et al.*, 1991) but it also metabolizes 2-AG (Kozak *et al.*, 2000) to prostaglandin E_2–glycerol ester (PGE_2-G). COX-2 expression is induced by various inflammatory and other injurious stimuli and is a major producer of prostaglandins during an inflammatory response (Masferrer *et al.*, 1994). PGE_2-G is a multifunctional signalling molecule whose effects include immune system modulation, hyperalgesia and enhanced neuronal activity (Sang and Chen, 2006; Sang *et al.*, 2006; Hu *et al.*, 2008). COX-2 inhibition was also shown to prolong DSI in hippocampal slices (Kim and Alger, 2004), suggesting that it is involved in limiting the retrograde signalling actions of 2-AG. A subsequent study in cultured hippocampal neurons identified a subpopulation of inhibitory interneurons in which the duration of DSI was determined by both MAGL and COX-2 (Straiker and Mackie, 2009). These results suggest that in some inhibitory neurons, COX-2 and MAGL cooperatively determine the duration of DSI, whereas in other inhibitory neurons, MAGL may be the dominant 2-AG degrading enzyme.

CNS insults such as ischaemia, trauma and seizures all lead to COX-2 induction (Lapchak *et al.*, 2001; Takemiya *et al.*, 2003). Interestingly, in cultured autaptic hippocampal neurons, overexpression of COX-2 with endogenous MAGL shortened the duration of DSE by almost a half (Straiker *et al.*, 2011). Additionally, because 2-AG production during CNS insults can be neuroprotective (Sinor *et al.*, 2000; Panikashvili *et al.*, 2001), induction of COX-2 may enhance neurotoxicity, both by decreasing 2-AG levels and by transforming 2-AG into the excitatory neuromodulator PGE_2-G. Taken together, these data demonstrate that COX-2 can profoundly influence 2-AG signalling.

Interestingly, the products of 2-AG metabolism by COX-2 generally oppose CB_1 receptor-mediated 2-AG functions. COX-2 oxygenation of 2-AG has been implicated in glutamate-induced excitotoxicity (Sang *et al.*, 2006), through PGE_2-G activation of caspase 3, ERK, p38 mitogen-activated protein kinase, IP_3 and NF-κB signalling (Sang *et al.*, 2007). Taken together, the data suggest that antagonism of the PGE_2-G receptor or novel inhibitors of COX-2-mediated PGE_2-G formation could be used to treat neurodegenerative and inflammatory diseases. Recent studies demonstrate that it is possible to develop COX inhibitors that preferentially inhibit oxygenation of 2-AG (Duggan *et al.*, 2011). For example, lumiracoxib inhibited COX-2 oxygenation of 2-AG, without affecting oxygenation of AA whereas celecoxib inhibited AA oxygenation more effectively than that of 2-AG (Duggan *et al.*, 2011). This selective inhibition of 2-AG oxygenation appears to be therapeutically beneficial, as it was efficacious in a mouse model of anxiety (Hermanson *et al.*, 2013) and would avoid the effects of impaired prostaglandin formation.

While less well studied, a number of lipoxygenases can metabolize 2-AG, producing hydroperoxy derivatives of 2-AG (Kozak and Marnett, 2002). In COS-7 cells, arachidonate 12-lipoxygenase was shown to convert 2-AG into the 2-glyceryl ester of 12(*S*)-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (Moody *et al.*, 2001). However, these mechanisms remain to be studied in detail or *in vivo*, especially with regard to their significance in the CNS.

FAAH – another enzyme that can break down 2-AG?

FAAH is a dimeric integral membrane protein. Although it is found throughout the body, it is most active in the brain and liver. In brain homogenates, hippocampus and cortex appear to have the highest FAAH activity (Thomas *et al.*, 1997). The enzyme degrades a variety of fatty acid amides, including AEA (Cravatt *et al.*, 1996). This broad substrate specificity must be

considered when interpreting the results of experiments using FAAH inhibition. In the brain, FAAH immunoreactivity is primarily neuronal and is enriched in somata and dendrites (Egertova *et al.*, 2003), where it is primarily found to be associated with the cytoplasmic face of smooth ER, mitochondria and, less frequently, the cell membrane (Gulyas *et al.*, 2004). Similarly, in cultured hippocampal neurons, FAAH staining was exclusive to neurons and was primarily present in somata and proximal dendrites (Straiker *et al.*, 2011). This staining pattern was in concordance with FAAH lacking rapid effects on synaptic transmission, as the FAAH blocker URB597 did not affect the time course of DSE (Straiker *et al.*, 2011). FAAH can hydrolyse 2-AG *in vitro*, although the consequences of this *in vivo* appear limited (Goparaju *et al.*, 1998). For example, FAAH knockout and FAAH inhibitors generally do not alter 2-AG levels (Lichtman *et al.*, 2002; Kathuria *et al.*, 2003; Schlosburg *et al.*, 2010). Moreover, FAAH knockout did not desensitize CB_1 receptors (Straiker and Mackie, 2005), in contrast to MAGL knockout, which caused profound CB_1 receptor desensitization (Marrs *et al.*, 2010; Schlosburg *et al.*, 2010). However, in autaptic hippocampal cultures, overexpression of FAAH with endogenous MAGL did shorten the duration of DSE (Straiker *et al.*, 2011). In summary, FAAH does not appear to play a role in degrading synaptically released 2-AG in the systems (short-term synaptic plasticity) discussed above; however, if FAAH expression is strongly up-regulated, it may participate.

2-AG phosphorylation and acylation as clearance mechanisms

Lipid kinases with activity against MAG can phosphorylate 2-AG to generate 2-arachidonoyl-LPA (2A-LPA) (Nakane *et al.*, 2002), which is an agonist for LPA receptors (LPA_1-LPA_6) (Choi *et al.*, 2010), and an important signalling molecule in its own right. This modification will decrease 2-AG, attenuating CB_1-receptor-mediated effects, but it will also have the consequence of increasing LPA-mediated signalling. 2A-LPA can also be converted back to 2-AG by lipid phosphatase(s) (Nakane *et al.*, 2002), which provides an alternative route for 2-AG synthesis. One LPA kinase is the multi-substrate lipid kinase (Waggoner *et al.*, 2004), also called acylglycerol kinase (Bektas *et al.*, 2005). Whereas acylation of MAG to a DAG is a theoretical pathway for decreasing 2-AG bioavailabilty, neither of the two cloned monoacylglycerol acyltransferases, MGAT1 (Yen *et al.*, 2002) or MGAT2 (Cao *et al.*, 2003), are expressed at detectable levels in the CNS. The 2-AG/2A-LPA/LPA cycle demonstrates that inter-conversion of neuromodulators may be an economical means for a cell to simultaneously regulate two signalling systems – by removing an effector from one signalling system and in the process converting it into an effector for another signalling system.

Why do neurons have so many 'options' for degrading 2-AG?

The diversity of enzymes involved in terminating 2-AG signalling allows fine-tuning of this pathway, both spatially and state-dependently (e.g. following ischemia). In the simplest view, 2-AG is synthesized in the post-synaptic cell. If large amounts of 2-AG are produced, it may be post-synaptically degraded by ABHD6 into AA and glycerol. The remaining 2-AG diffuses across the synapse, interacting with CB_1 receptors on the pre-synaptic terminal. Pre-synaptically, 2-AG can be degraded by MAGL, COX-2 or ABHD12. A summary of this arrangement is depicted schematically in Figure 1. Depending on the amount of 2-AG produced, and the enzymes involved, the duration and spatial spread of 2-AG can be controlled and additional modulators (e.g. PGE_2-G and 2A-LPA) produced.

The last decade has seen many exciting advances in the cannabinoid field. One of them is that 2-AG has emerged as the chief endocannabinoid neuromodulator. Moreover, the field as a whole has moved from brute force manoeuvres to either activate or suppress cannabinoid receptors to more subtle ways of fine-tuning their signalling. One way to achieve this goal is by manipulating 2-AG production or degradation. The various enzymes synthesizing or degrading 2-AG appear to be rich targets for pharmacological manipulation in a variety of disease states. This is not only due to their manipulation affecting 2-AG levels but also because the metabolic products of 2-AG can themselves have significant biological activity.

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Conflict of interest

None.

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